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Detection, Shedding, and Environmental Contamination

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Chronic wasting disease (CWD) of deer and elk is unique among the transmissible spongiform encephalopathies. Our long-term goal is to better understand the epidemiology of CWD and thus develop strategies for management and control. The specific goals of these studies are to develop sensitive assays for PrP ^{res} as a marker for infectivity, and use these techniques to monitor the dynamics and modes of shedding of PrP ^{res} from orally infected mule and white-tailed deer and elk. Finally these techniques will be applied to investigating the nature of environmental contamination that may be associated with CWD transmission. Protease resistant prion protein from brains of CWD affected deer and elk (PrP ^{res}) were purified and used in a variety of detection assays. PrP ^{res} was detected using antibody based techniques but not, as of yet, by potentially more sensitive detection methods. Deer and elk have been obtained and the infrastructure is in place to begin the studies of CWD shedding in vivo. In addition, in support of investigations of environmental contamination by the CWD agent, we have mapped areas of high, moderate, and low CWD contamination at two CWD endemic facilities.			
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INTRODUCTION

Chronic wasting disease (CWD) of deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) is unique among the transmissible spongiform encephalopathies (TSEs) in that it occurs in free-ranging as well as captive wild ruminants and environmental contamination appears to play a significant role in maintenance of the disease. The precise modes of transmission of CWD are not known although we have shown that horizontal transmission and environmental contamination associated with excreta and carcasses may occur (Miller et al., 2004). But maternal transmission does not appear to play a significant role (Miller and Williams, 2003) in maintenance of CWD in cervid populations. Our long-term goal is to better understand the epidemiology of CWD and apply that information to development of strategies for management and control. To that end we are investigating the dynamics and modes of CWD agent shedding from infected mule deer, white-tailed deer, and elk. The approach includes experimentally infecting cervids, serial collections of a variety of biological samples, and assay of these materials by various means to attempt to detect protease resistant prion protein (PrP^{res}). In addition, because of the concern about environmental contamination associated with excreta, we will be collecting and assaying a variety of environmental specimens collected from areas of presumed high, moderate, and low contamination in CWD endemic facilities.

BODY

Aim 1: Develop analytical tools to detect PrP^{CWD} in excreta, blood, and environmental samples.

The goal of Aim 1 of the grant proposal is to develop analytical tools to detect the pathologic form of prion protein associated with chronic wasting disease (PrP^{CWD}) in excreta, blood, and environmental samples. Efforts in the Lewis laboratory were directed primarily towards this goal. We have purified PrP^{CWD} and the cellular isoform of prion protein (PrP^C). PrP^{CWD} was purified from brains of CWD-affected elk (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) using methods described by Bolton et al. (1982) and Kocisko et al. (1994). Initial purification efforts were hampered because the brains of animals with CWD used for purification had only small amounts of PrP^{CWD} and we were unable to adequately identify brains from CWD-affected animals with large amounts of PrP^{CWD}. This problem was overcome by screening brains from CWD-positive animals diagnosed by immunohistochemistry using a rapid screening assay (enzyme-linked immunosorbent assay [ELISA], BioRad, Hercules, California) and brains with the highest amounts of PrP^{CWD} were identified and used for PrP^{CWD} purification. Our purification protocol uses detergent extraction of PrP^{CWD} from brain homogenates followed by ultracentrifugation to isolate and concentrate the protein. In total approximately 120 µg of both elk and white-tailed deer PrP^{CWD} were obtained.

Purified PrP^{CWD} was characterized biochemically by proteinase K digestion and western blot analysis. Results indicated that the purified PrP^{CWD} was partially PK resistant showing the characteristic 6 to 7 kD size shift. In addition pathologic prion protein (PrP^{Sc}) was purified from the brains of hamsters infected with the 263K strain of rodent-adapted scrapie for use in preliminary PrP^{CWD} detection method development. Approximately 1 mg of 263K PrP^{Sc} was obtained from the purification. The yield of PrP^{Sc} from 263K infected hamster brain is typically higher, at roughly 50 µg per gram brain starting material, than that for CWD affected cervid

brain which is on the order of 5 µg per gram brain starting material (G. Raymond, personal communication). Initially, 263K purified PrP^{Sc} will be used in place of the purified PrP^{CWD} for assay development to minimize time lost to reagent preparation followed by application of these techniques to PrP^{CWD}.

The purity of purified PrP^{CWD} and PrP^{Sc} was assessed by SDS/PAGE with silver staining and western blotting and protein quantitation by bicinchoninic acid (BCA) assay. Silver stain analysis indicated there were a number of impurities present in all of our purified PrP preparations. BCA assay combined with western blot quantification confirmed the presence of impurities with total protein concentrations of 2.2 and 3.4 mg/ml respectively for elk and WTD PrP^{CWD} purified stock solutions compared to the 0.120 mg/ml PrP concentrations respectively for the same stock solutions. The purified hamster PrP^{Sc} also contained impurities as demonstrated by SDS/PAGE with silver staining, Western blot, and BCA assay. These impurities are not expected to interfere with current PrP^{res} detection methodologies currently being developed as described below. Therefore, chromatographic purification methodologies described in the grant proposal have not been employed and are not expected to be needed at this time.

Purification of the normal cellular form of PrP^C will be performed using *E. coli* derived recombinant PrP clones for several cervid PrP genotypes. Clones containing the full PrP sequence, including the N-terminal trafficking sequence from residues 1 to 22 and the C-terminal GPI-anchor sequence from residues 240 to 256 of the cervid PrP sequences were obtained from Alex Bossers (Central Institute for Animal Disease Control, Lelystad, The Netherlands). These clones were used previously for expression in mammalian cell culture and therefore have the N- and C-terminal signaling sequences (Raymond et al., 2000). We have subcloned the PrP sequence from residues 23 to 239 via polymerase chain reaction (PCR), restriction enzyme digest and ligation into a pET30a expression vector for protein expression in *E. coli*. We are currently awaiting the results of sequencing analysis of the ligated pET 30 vector for confirmation of successful ligation of the PCR amplified PrP insert into the pET vector. Once we confirm the DNA sequences we will transform the white-tailed deer, mule deer, and elk PrP insert containing pET vector DNA into BL21(DE3) *E. coli*, grow up individual colonies with isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and then purify the expressed PrP protein via immobilized metal affinity chromatography using a nickel charged sepharose using previously described methods (Rezaei et al., 2000; Speare et al., 2003). Purified PrP^C protein will be used for developing the analytical specificity of our PrP^{res} detection methods and will be a source of large quantities of PrP readily usable as a detection target protein.

Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry was performed on the purified PrP^{CWD} described above. Initial efforts were unsuccessful due to difficulty resolving a clear peak associated specifically with PrP^{CWD}. We believe the lack of a clearly resolved peak is due to the sample preparation method. Specifically, we believe the method used did not monomerize the PrP^{res} aggregate effectively. Upon investigating PrP aggregate sample preparation further we obtained methods to effectively monomerize PrP^{res} and prepare it for successful mass spectrometry (A. Hughson, personal communication). A nanospray ionization source and associated hardware (including cameras, monitors, micro ion spray kit) and related software (Analyst 1.3) for our API 2000 (LC/MS/MS, Applied Biosystems, Foster City, California) have been obtained but are not yet functional.

Given the relative difficulty we have had in getting adequate PrP^{res} and time necessary to prepare PrP^{res} for mass spectrometric analysis, we have also investigated an alternate method for detecting proteins at attomolar levels (six orders of magnitude more sensitive than the typical sensitivity for mass spectrometry) using the bio-bar code assay (BBCA) (Nam et al. 2003; Nam et al. 2004). The assay is rapid, taking hours to complete, requires no major investment in instrumentation unlike mass spectrometric-based methods and is adaptable to high throughput applications. The BBBCA uses a target specific antibody bound to a magnetic microparticle to bind the target protein of interest followed by the binding of a gold nanoparticle hybridized with a different antibody specific to the same target protein and bar code DNA. This bar code DNA can be eluted and detected by either a PCR or PCR-less methodology (Nam et al. 2003; Nam et al. 2004). We have started a collaboration with the group that developed the BBBCA, led by Dr. Chad Mirkin at Northwestern University. Using BBBCA probes containing the monoclonal antibody 6H4 (Epitope 147-155 cervid PrP; Prionics, Schlieren, Switzerland) and the polyclonal antibody R35 (epitope 85-95 of cervid PrP; generously provided by Dr. Byron Caughey, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana) we have been unable to reliably detect PrP^{res} from cervids from any sample. We have changed many parameters in an attempt to eliminate the possibility of procedural or sampling errors and had no success. We believe the lack of detectable signal is due to an incompatibility with the 6H4 and R35 antibodies. We have sent different antibodies, which were obtained commercially and from other collaborators, to the Mirkin group and are planning to test them in August, 2004. We are in the process of generating our own monoclonal and polyclonal PrP specific antibodies that we plan to use for the BBBCA.

Methods for concentrating environmental samples have been investigated. Initial experiments have used detergent extraction of PrP^{res} from feces. The anionic detergent N-lauroyl sarcosyl was used at a 10% (w/v) concentration for all extractions. Extractions using 10% N-lauroyl sarcosyl were performed on feces from uninfected, clinically CWD infected, and clinically CWD infected spiked with brain homogenate from CWD affected white-tailed deer. Extracts were treated with proteinase K and then with phosphotungstic acid to precipitate PrP^{CWD} as described by others (Wadsworth et al., 2001). Precipitates were separated by centrifugation and analyzed by SDS/PAGE with western blotting. Results indicated that PrP^{res} in the spiked samples is extractable but there are no obvious PrP bands present in any samples from CWD affected animals. Bands are present at higher apparent molecular weight in fecal extracts from clinically affected deer that are not present in extracts of feces from uninfected deer. Experiments are underway to try and elucidate whether these bands are aggregated forms of PrP. Fecal samples from CWD affected deer and elk were also assayed by a TSE-specific ELISA (Bio-Rad) routinely used for CWD tissue assays, with results not distinguishable from negative controls.

Because we have not yet developed adequate detection systems for quantities of PrP^{CWD} in biological or environmental samples, in accordance with the grant proposal, we have developed collaborative arrangements with Dr. Michael Oldstone (The Scripps Research Institute, San Diego, California) and Dr. Bruce Chesebro (Rocky Mountain Laboratories) to assay biological samples from CWD infected cervids in transgenic mice expressing white-tailed deer PrP^C. These mice were generated using PrP^C open reading frame (ORF) from a white-tailed deer which was cloned, sequenced, and used to replace the mouse PrP^C ORF. PrP^C is expressed

by these mice (M. Oldstone, personal communication) and some have been inoculated with brain material from cervids with CWD supplied by our laboratory.

The collaboration with Greg Raymond (Dr. Byron Caughey's Laboratory, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana), as suggested by reviewers of our original proposal, has been very useful and Dr. Jonathan Speare (Postdoctoral Research Associate, Department of Molecular Biology, University of Wyoming) has spent time on two occasions over the last year working in Dr. Caughey's laboratory.

Aim 2. Evaluate multiple biological samples collected from experimentally infected mule deer, white-tailed deer, and elk throughout the CWD incubation period.

Funding for this project was granted in August 2003 well after the time frame for obtaining deer and elk for use in this study in 2003. As indicated in the grant proposal, the ability to obtain wild animals for research is highly seasonal. This strict seasonality is due to the fact that neonatal fawns must be bottle-raised so that the animals will be tractable enough to be handled for collection of biological samples. In addition, the infrastructure to conduct the biological sampling (metabolic chambers) had to be ordered, built, renovated, or installed. We are now at a point where animals will be inoculated and biological samples collected beginning fall 2004.

Colorado: We began preparations for experimentally infecting and sampling mule deer and white-tailed deer fawns at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (FWRF, Fort Collins, Colorado) in support of Aim 2. During Oct 2003–Jul 2004, we repaired, modified, and improved FWRF facilities for holding and sampling up to 30 mule deer and 30 white-tailed deer for upcoming sample collections. Facility modifications to accommodate this work included construction of four new outdoor paddocks (~0.1 ha/ea) with dedicated electricity, automatic watering troughs, shelters, and feed storage sheds; all paddocks were double-fenced, and species separated by a lined drainage trough. We also constructed alleyways and ramps, and cleaned and refurbished existing metabolic cages for use in sample collections; cages were resurfaced with a urethane coating to allow cleaning and disinfection between sampling sessions.

In addition to facility modifications, we began bottle-raising and training mule deer and white-tailed deer fawns for use as experimental subjects in May 2004. As of 11 August 2004, we were maintaining 32 mule deer fawns (16 males and 16 females) and 16 white-tailed deer fawns (eight males and eight females) for experimental use beginning in fall 2004; eight additional white-tailed deer fawns are being hand-raised by cooperators in Kansas. If adequate numbers of white-tailed deer fawns are not available this fall we will likely require a second, smaller rearing effort in summer 2005 to augment sample sizes. We began training fawns to tolerate confinement in metabolic cages in July 2004; because fawns have readily adjusted to the cages, progress in training has proceeded ahead of schedule. In August, blood samples were collected for *Prnp* genotype determination; those assays are pending.

Wyoming: We began preparations for experimentally infecting and sampling elk in fall 2003 through summer 2004. Ten metabolic chambers large enough to hold adult elk were built

and installed at the Wyoming Game and Fish Department's Sybille Wildlife Research Unit (Sybille, Wheatland, Wyoming) along with chutes, alleyways, and corrals for linking these chambers. Twenty-two elk calves are available to be orally inoculated in fall 2004. Blood collections for *Pnnp* genotyping will be collected at the time of inoculation.

Facilities for control cervids were prepared as described above at the University of Wyoming's Red Buttes Research Center (Laramie, Wyoming). Metabolic cages have been built and installed along with chutes and runways for handling the animals. Eight mule deer (five females and three males) and three white-tailed deer (two females and one male) are available for sampling. Control elk will be captured from the wild during the winter. Additional infrastructure to support Aim 2, installation of a freezer and development of a sample and tissue archival system are in place.

Aim 3. The goal of this Aim is to determine if PrP^{res} can be detected in samples collected from facilities contaminated with the CWD agent.

Both CWD endemic facilities, FWRP (Figure 1) and Sybille (Figure 2), have been qualitatively evaluated based on history and pen and pasture usage by CWD-affected animals and categorized into areas regarded as having low, moderate, or high probability of surface contamination with CWD agent. At Sybille, 6.8, 5.4, and 1.5 ha are considered areas of low, moderate, and high degree of contamination, respectively. These maps will be used to design a replicated transect system to collect soil, vegetation, and invertebrate and vertebrate samples to support the goals of this Aim.

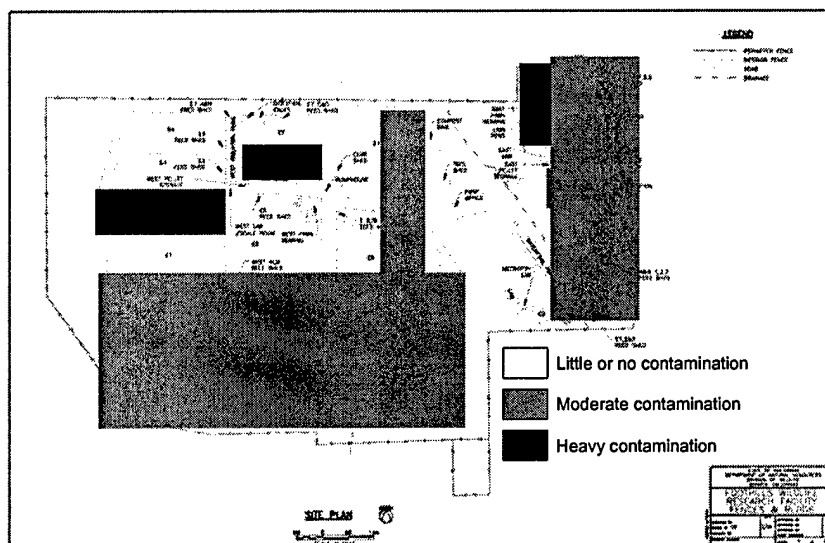


Figure 1. Qualitative assessment of areas of low, moderate, and high CWD-associated surface contamination at the Colorado Division of Wildlife's Foothills Wildlife Research Facility, Fort Collins, Colorado.



Figure 2. Qualitative assessment of areas of low, moderate, and high CWD-associated surface contamination at the Wyoming Game and Fish Department's Sybille Wildlife Research Unit. Wheatland, Wyoming.

KEY RESEARCH ACCOMPLISHMENTS

- PrP^{CWD} from white-tailed deer and elk has been purified.

REPORTABLE OUTCOMES

None at this time.

CONCLUSIONS

We are still in the early stages of the proposed work primarily due to timing of the granting period relative to the biology of the deer and elk to be used in these studies. Progress is being made in purifying PrP^C and PrP^{res} from tissue and fecal samples but we have not yet been able to detect PrP^{res} from tissue, fecal, or environmental samples using MALDI-TOF, BBCA, western immunoblot, or ELISA. We still have not exhausted all the proposed approaches to preparation and concentration of samples for detection of PrP^{CWD}. As an alternative, as originally proposed, we are investigating use of transgenic mice expressing white-tailed deer PrP^C to assay samples for the presence of PrP^{CWD} through collaborative arrangements. Animal facilities and other infrastructure are nearly completed and animals have been obtained so that CWD inoculations and sample collections will begin this fall.

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